Mutation of a Consensus Purine Nucleotide Binding Site in the Adeno-Associated Virus *rep* Gene Generates a Dominant Negative Phenotype for DNA Replication

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Adeno-associated virus (AAV) contains a multifunctional nonstructural gene, *rep*, which is required for AAV DNA replication and has pleiotropic effects on positive and negative regulation of gene expression. All of the parvovirus nonstructural genes contain a region of highly conserved amino acid homology. Within this conserved region is the consensus sequence for a purine nucleotide binding site. We constructed a mutant AAV having a mutation in this site by converting lysine 340 to histidine. The resulting mutant AAV genome, pNTC23, overproduced the mutant Rep proteins, indicating that these proteins are autoregulated. Furthermore, the mutant gene was unable to replicate but was able to inhibit in *trans* wild-type AAV DNA replication. Thus, pNTC23 represents a dominant negative mutant of AAV. These results suggest that *rep* has separate functional domains important for DNA replication.

Site-specific mutagenesis can be used readily to generate mutations in specific genes. Generally, this results in inactivation of the gene product. However, some mutations may result in overexpression of a mutant polypeptide which can inhibit the activity of the wild-type gene product. Recently, it was pointed out that such dominant negative mutations may be especially useful for the analysis of regulatory proteins or enzymes which have several functional domains or activities (22). We have constructed a dominant negative mutation in the adeno-associated virus (AAV) *rep* gene.

AAV, like all other parvoviruses, contains a multifunctional gene, *rep*, which is required for AAV DNA replication (7, 8, 21, 31, 33, 35) and has pleiotropic effects on positive and negative regulation of gene expression (25, 26, 36, 38). The AAV *rep* gene uses two transcription promoters to encode four overlapping Rep proteins: Rep78 and Rep68 are encoded by transcripts from the *p*₅ promoter, and Rep52 and Rep40 are encoded by transcripts from the *p*₁₉ promoter (29, 38). Rep78 and Rep52 are encoded by unspliced mRNAs, whereas Rep68 and Rep40 have altered carboxyl termini, reflecting splicing of the transcripts (29, 38). Rep78 or Rep68 is required for AAV replicating-form (RF) DNA replication apparently by binding to the replication origin (1, 24). Rep52 and Rep40 are not required for RF DNA replication but may be required for the accumulation of single-stranded DNA (ssDNA) and infectious particles (8).

All of the parvovirus nonstructural genes contain a region of highly conserved amino acid homology (2, 9, 32). Notable within this region is the consensus sequence G(X)₄GKT/S(X)₅₋₆I/L/V for a purine nucleotide binding site present in a variety of other eucaryotic and procaryotic proteins, including the large T antigens of simian virus 40 and polyomavirus (2, 5, 9, 13, 16, 19). In simian virus 40 and polyomavirus an ATPase activity that is associated with this domain is required for viral DNA replication (10-12).

In AAV the putative purine nucleotide binding site (2, 33) is common to all four Rep proteins. In this work, we constructed a mutant AAV having a mutation in this site.

The codon for lysine 340 in the consensus nucleotide binding sequence was changed to histidine. The resulting mutant AAV genome (pNTC23) overproduced the mutant Rep proteins but was unable to replicate. The mutant gene was able to inhibit in *trans* wild-type AAV DNA replication. Thus, pNTC23 represents a dominant negative mutant of AAV.

MATERIALS AND METHODS

Cells and viruses. An established human embryonic kidney cell line transformed by adenovirus 293-31 (293 cells; 18) was grown at 37°C in 5% CO₂ in monolayer cultures in Eagle minimal essential medium supplemented with antibiotics and 5% fetal calf serum. Human adenovirus type 5 and AAV type 2 (AAV-2) were grown and assayed as described previously (6).

Plasmid construction and mutagenesis. Plasmid construction and mutagenesis were performed as described in detail elsewhere (7, 8). Plasmid pNTC244 contains the wild-type AAV-2 genome inserted via *Bgl*III linkers (27) at the *Bam*HI site in the polylinker sequence of the phagemid pTZ19U (8). pNTC3 is an amber mutant of pNTC244 having a point mutation in the *rep* gene at map position 22 (7). pNTC23 was generated from pNTC244 by oligonucleotide site-directed mutagenesis of the wild-type AAV-2 genome with the oligonucleotide 5'-ACTACCGGGCACACCAACATC-3' by use of the Muta-gene system (BioRad Laboratories, Richmond, Calif.) as previously described (7, 8). pNTC41 was produced by deleting the *Sst*I fragment between the *Sst*I sites in the polylinker of pNTC244 and at nucleotide 814 (map position 17.4) of the AAV-2 sequence (8). This deletion removes the *p*₅ transcription unit but leaves the *p*₁₉ transcription unit intact. pNTC41a was generated from pNTC41 by introducing the same amber mutation as in pNTC3 (8). pNTC8 was constructed from pNTC23 by deleting the *p*₅ transcription unit by use of the *Sst*I sites as described for pNTC41. pTS18 is a Rep⁻ *p*₄₀-*cat* construct (36, 37) grown in *Escherichia coli* HB101 and purified by standard procedures (28, 36). Stocks of pTZ19U and the pNTC plasmids were grown in *E. coli* MV1190 and purified by alkaline lysis and

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FIG. 1. AAV *rep* gene structure and consensus purine nucleotide binding site sequence. The AAV-2 genome (33) is shown on a scale of 100 map units (1 unit is approximately 47 nucleotides). Control regions are shown as stippled boxes (DNA replication origins; 20) and solid circles (p_5 , p_{19} , and p_{40} transcription promoters). Heavy arrows show p_5 or p_{19} mRNA, with the intron (map units 41 to 48) shown by the carets and the polydenylation site at map unit 96 shown by the arrowheads. The p_{40} transcripts which code for capsid proteins and the minor RNA species derived by alternate splicing are omitted for clarity. The coding regions for the four proteins, Rep78, Rep68, Rep52, and Rep40 (29, 38), are shown by open rectangular boxes. The putative purine nucleotide binding region with consensus amino acids underlined (2) at map position 28.6 is indicated at the top, as are nucleotides 1338 and 1340, which were altered to convert the consensus lysine 340 codon AAG to a histidine codon, CAC. In the lower portion are shown the regions of AAV-2 DNA contained in several plasmids. pNTC244, pNTC23, and pNTC8 contain the entire AAV-2 genome. In pNTC41, pNTC41a, and pNTC8, nucleotides 1 to 814 of AAV-2 are deleted. pNTC23 and pNTC8 contain the mutation in the purine nucleotide binding site indicated by $\times$. pNTC3 and pNTC41a contain a nonsense mutation (indicated by "a") at nucleotide 1033 generated by changing a serine codon, UCG, to an amber codon, UAG (7). The nucleotide numbering scheme is that of Srivastava et al. (33). kb, Kilobases.

pZ523 columns (5 Prime-3 Prime, Inc., West Chester, Pa.) in accordance with manufacturer protocols (7, 8, 28).

DNA transfection and viral DNA replication. 293 cells (5×10^5 /35-mm dish) were transfected by the calcium phosphate precipitation method (35). For AAV replication, the cells were infected with adenovirus type 5 (5 PFU per cell) 1 h prior to transfection as described before (27, 35). For individual transfection experiments, the total amount of DNA in each transfection mixture was adjusted to the same level with control plasmid pTZ19U.

Intracellular AAV DNA replication forms were extracted 40 h after transfection by the modified Hirt sodium dodecyl sulfate-high-salt lysis procedure (27, 35). The DNA forms were analyzed by agarose gel electrophoresis followed by blotting to nitrocellulose filters and hybridization with [32 P]AAV probes radiolabeled by the random priming procedure (17). The amount of radioactivity hybridized was quantitated by scintillation counting of individual bands from the blots.

Protein analysis. Cell lysates for analysis of Rep proteins were prepared 48 h after transfection of adenovirus-infected

293 cells and electrophoresed in 12% polyacrylamide gels. AAV Rep proteins were detected with anti-Rep antisera by the immunoblotting procedure (29, 38) exactly as described previously (8).

CAT assays. 293 cells (10^6) were transfected with 1 μ g of pTS18, a Rep⁻ AAV p_{40} -cat vector (36, 37), and up to 10 μ g of a complementing plasmid. Cell extracts were prepared at 48 h after transfection and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (36).

RESULTS

Mutagenesis of the putative nucleotide binding site in the *rep* reading frame. The structure of the AAV genome and the organization of the *rep* gene are shown in Fig. 1. The *rep* gene is transcribed from two different promoters, designated at p_5 and p_{19} (at map positions 5 and 19, respectively). Four Rep proteins were identified with specific anti-Rep antibodies (29, 38). The predominant proteins were Rep78 and

FIG. 2. Production of Rep proteins by mutant AAV genomes. Adenovirus-infected 293 cells (5×10^5 cells per 35-mm dish) were transfected with the AAV plasmids designated by the numbers at the top (the pNTC designation is omitted). Ad indicates control adenovirus-infected cells. Protein extracts were analyzed by immunoblotting with an anti-Rep antibody.

Rep52, whereas Rep68 and Rep40 were produced at much lower levels (29, 38; Fig. 2).

The consensus purine nucleotide binding site in the *rep* reading frame (2) was at map position 28.6 and was thus common to all four Rep proteins (Fig. 1). To produce a mutation in the putative nucleotide binding site, we introduced a two-base change in the lysine 340 codon (Fig. 1). Thus, the codon AAG was converted to CAC (nucleotides 1338 and 1340), coding for histidine, to generate mutant pNTC23.

Several other mutant AAV plasmids are also diagrammed in Fig. 1. pNTC3 contains an amber mutation at map position 22. pNTC41, pNTC41a, and pNTC8 are analogous to pNTC244, pNTC3, and pNTC23, respectively, but retain only the p_{19} transcription unit.

Rep protein production by AAV mutants. The expression of the Rep proteins from mutant and wild-type AAV genomes was determined by immunoblot analysis of extracts prepared from adenovirus-infected 293 cells transfected with the appropriate plasmids. pNTC23 produced all four Rep proteins at a higher level than did the wild-type AAV plasmid pNTC244, suggesting that the production of Rep proteins is autoregulated (Fig. 2). It is possible that the overproduction by pNTC23 represents an increased stability of Rep proteins. However, we have previously shown by pulse-chase analysis that Rep proteins are highly stable (30). Figure 2 also shows, as we reported previously for pNTC41 and analogous plasmids (8, 29, 38), that mutation or deletion of the p_5 promoter or the amino terminus of Rep78 or Rep68 resulted in the overproduction of Rep52 and, to a lesser extent, of Rep40. In contrast, pNTC8, which is analogous to pNTC41 but which has the same mutation in the consensus nucleotide binding site as pNTC23, did not overproduce Rep52. This result suggests that the expression of Rep52 (or Rep40) may be independently autoregulated in addition to being regulated by Rep78 or Rep68. Elsewhere we showed that pNTC3 and pNTC41a, which have an amber mutation at map position 22, produced no Rep proteins (7, 8).

Blockage of AAV DNA replication by a mutation in the consensus nucleotide binding site. The Rep proteins are essential for AAV DNA replication (7, 8, 21, 24, 31, 35). The effect of the mutation in the putative nucleotide binding site of *rep* was analyzed by isolating intracellular replicating AAV DNA from adenovirus-infected 293 cells transfected with pNTC23 or pNTC244. pNTC23 was defective for

FIG. 3. Replication of AAV genomes. Adenovirus-infected 293 cells were transfected with pNTC244 or pNTC23 DNA as follows. Intracellular AAV DNA was extracted, electrophoresed in an agarose gel, and detected by Southern blotting with an AAV [32 P]DNA probe as described in Materials and Methods. Lanes: 1, 6 μ g of pTZ19U; 2, 0.1 μ g of pNTC23; 3, 1 μ g of pNTC23; 4, 6 μ g of pNTC23; 5, 0.1 μ g of pNTC244; 6, 1 μ g of pNTC244. All transfection mixtures were adjusted to 6 μ g with control plasmid pTZ19U. RFd, RFm, and SS indicate replicating dimer DNA, replicating monomer DNA, and progeny ssDNAs, respectively.

replication, and the normal AAV DNA intracellular species (4, 34), including monomer and dimer RFs, (RFm and RFd, respectively) and progeny ssDNA, were not observed (Fig. 3). We reported elsewhere that pNTC3, pNTC41, and pNTC41a were all unable to replicate AAV DNA (7, 8). Similarly, pNTC8, which is both an *ori* mutant and a *rep* mutant, like pNTC41 and pNTC41a, did not replicate AAV DNA (data not shown).

Inhibition of AAV DNA replication by mutant Rep proteins. Detection of AAV DNA replication in adenovirus-infected 293 cells is a sensitive assay, and RFs resulting from transfection of less than 10 ng of an AAV-containing plasmid can be observed (unpublished results). We used this assay to determine if the mutant Rep proteins from pNTC23 could inhibit wild-type DNA replication. AAV DNA replication was easily detected when 50 ng of wild-type AAV, pNTC244, was transfected into adenovirus-infected 293 cells (Fig. 4, lane 1). Increasing amounts of wild-type AAV plasmid increased the amount of intracellular DNA synthesis (Fig. 4, lanes 2 to 4). However, when 50 ng of pNTC244 was cotransfected with increasing amounts of pNTC23, AAV DNA replication was severely inhibited (Fig. 4, lanes 8 to 10). Indeed, significant inhibition by pNTC23 was seen with only a 1:1 ratio of pNTC23 to pNTC244 (Fig. 4, lane 8). When the amber mutant pNTC3 was cotransfected with pNTC244 (Fig. 4, lanes 5 to 7), RF DNA levels were decreased by no more than 2-fold, even at a 20-fold excess of the mutant. Since the amber plasmid pNTC3 supplies no Rep protein, the drastic inhibition by pNTC23 must have been due to the mutant Rep proteins and not to the effect of adding additional AAV templates containing replication origins.

Additional experiments (Fig. 5, lanes 7 to 12) showed that inhibition by pNTC23 was not observed when the amount of

FIG. 4. Inhibition of wild-type AAV DNA replication by a *trans*-dominant mutant. Adenovirus-infected 293 cells were transfected with 0.05 μ g of pNTC244 (lane 1) and cotransfected with an additional 0.05, 0.1, or 1 μ g of pNTC244 (lanes 2 to 4), pNTC3 (lanes 5 to 7), or pNTC23 (lanes 8 to 10), respectively. Lane 11 shows adenovirus-infected cells transfected with the control plasmid alone. All transfection mixtures were adjusted to 1 μ g with control plasmid pTZ19U. AAV DNA was extracted and analyzed as for Fig. 3. RFd, RFm, and ssDNA species are indicated. The additional species in lanes 7 and 10 are input pNTC3 and pNTC23 DNAs, respectively.

mutant plasmid was decreased to about one-tenth that of the wild-type plasmid. Similarly, inhibition could be overcome when the amount of wild-type plasmid was increased 10-fold (Fig. 5, lanes 2 to 5). Also, pNTC23 could be complemented for replication when wild-type *rep* was supplied by cotransfecting a 10- to 20-fold excess of an Ori⁻ AAV plasmid (data not shown).

The mutation in pNTC23 affected both the Rep78 or Rep68 and Rep52 or Rep40 reading frames. Thus, it was conceivable that the mutant Rep52 or Rep40 protein alone might inhibit wild-type AAV DNA replication. This possibility was tested by cotransfecting the wild-type AAV plasmid with pNTC8, as well as with pNTC41 and pNTC41a (Fig. 6). No significant inhibition of AAV DNA replication was observed with any of these plasmids, even at a 20-fold excess over the wild-type plasmid, pNTC244 (Fig. 6).

The data from experiments similar to those in Fig. 4 and 6 were quantitated by cutting the blots after hybridization and determining the amount of [³²P]DNA probe annealed to the RFm species. A 1:1 ratio of pNTC23 to pNTC244 produced 20-fold inhibition, whereas even a 20:1 excess of plasmids pNTC3, pNTC41, pNTC41a, and pNTC8 produced not more than 2- to 2.5-fold inhibition (Fig. 7). This lower inhibition by the latter plasmids may reflect simply a dilution effect of providing a large number of substrates (i.e., replication origins) that potentially bind Rep proteins. This appears to be the case even for pNTC3, which can be complemented by

FIG. 5. Effect of relative concentration of wild-type and mutant plasmids on AAV DNA replication. Adenovirus-infected 293 cells were transfected with DNA as follows: lane 1, 0.1 μ g of pNTC23; lanes 2 to 5, 0.1 μ g of pNTC23 plus 0.1 μ g (lane 2), 0.5 μ g (lane 3), 1 μ g (lane 4), or 2 μ g (lane 5) of pNTC244; lane 6, pTZ19U control DNA; lane 7, 0.05 μ g of pNTC244; lanes 8 to 12, 0.05 μ g of pNTC244 plus 0.1 μ g (lane 8), 0.05 μ g (lane 9), 0.01 μ g (lane 10), 0.005 μ g (lane 11), or 0.001 μ g (lane 12) of pNTC23. All transfection mixtures were adjusted to 2 μ g of total DNA with pTZ19U DNA. AAV DNA replication was analyzed as for Fig. 3.

the wild-type plasmid to generate mutant genomes that compete for, but do not supply, Rep proteins. Data similar to those in Fig. 7 were also obtained when the radioactivity in RFd or ssDNA was quantitated, except that ssDNA was more sensitive to inhibition than was RFd DNA (e.g., Fig. 4, lanes 5 to 7). This probably reflects a cascade effect in that decreased ssDNA accumulation may result both directly from decreased RFd DNA synthesis and perhaps indirectly from decreased wild-type protein synthesis. Both capsid proteins and the Rep52 or Rep40 protein are apparently required for efficient ssDNA accumulation (8, 21, 35).

***trans*-regulation by pNTC23-encoded Rep proteins.** Previous studies showed that in uninfected 293 cells the Rep proteins exert pleiotropic effects on the level of expression from chimeric *p*₄₀-*cat* constructs (36, 37). Thus, Rep proteins increased by two- to threefold the accumulation of chimeric *p*₄₀ RNA transcripts but inhibited the expression of CAT activity (37). The ability of the mutant Rep proteins from pNTC23 to *trans*-regulate *p*₄₀-*cat* expression was analyzed with the chimeric Rep⁻ *p*₄₀-*cat* construct pTS18 (Fig. 8) (36, 37). Cotransfection of wild-type pNTC244 with the reporter plasmid resulted in a fourfold inhibition of CAT activity (Fig. 8). In contrast, with pNTC23 there was about a twofold stimulation, whereas the *rep* amber mutant pNTC3 had a null effect (Fig. 8). This result suggests that the mutant pNTC23 may have retained the *trans*-activation property but apparently has lost the negative regulatory effect of *rep*.

DISCUSSION

Alteration of the putative purine nucleotide binding site in the AAV *rep* gene resulted in a mutant, pNTC23, which overproduced all four Rep proteins but was defective for AAV DNA replication. Furthermore, the mutant proteins

FIG. 6. Replication of wild-type AAV DNA in the presence of mutant AAV genomes producing only p_{19} -encoded Rep proteins. Adenovirus-infected 293 cells were transfected with 0.05 μ g of pNTC244 (lanes 1 to 4 and 6 to 14) and cotransfected with an additional 0.05, 0.1, or 1 μ g, respectively, of pNTC244 (lanes 2 to 4), pNTC41 (lanes 6 to 8), pNTC41a (lanes 9 to 11), or pNTC8 (lanes 12 to 14) or 1 μ g of control plasmid pTZ19U (lane 5). All transfection mixtures were adjusted to 1 μ g with the control plasmid. AAV DNA replication was analyzed as for Fig. 3.

were inhibitory for wild-type AAV DNA replication *in trans*. However, this inhibition was overcome by a 10-fold excess of wild-type AAV genomes. Presumably, this result reflects the need for increased wild-type Rep protein in the presence of overproduced mutant protein. pNTC23 fulfills the criteria for a dominant negative phenotype (22), suggesting that the putative purine nucleotide binding site may be important both for AAV DNA replication and for some of the pleiotropic properties of *rep*.

The Rep78 or Rep68 protein is necessary (7, 21, 31, 35, 36) and sufficient (8) for *in vivo* amplification of AAV duplex RF DNA. Consistent with this, Rep78 or Rep68 binds specifically to the AAV palindromic termini which constitute the replication origins (1, 24). Furthermore, recent evidence from *in vitro* assays indicates that the Rep78 or Rep68 protein appears to contain an ATP-dependent, site-specific, strand-specific nuclease activity involved in the resolution of the AAV DNA termini (32a). In *in vitro* assays, resolution of the termini but not binding of Rep78 or Rep68 to the origins requires ATP (24; Snyder et al., *in press*). Thus, the DNA-negative phenotype of pNTC23 provides *in vivo* evidence consistent with the possibility that a functional ATP binding site in Rep78 or Rep68 is required for Rep function in AAV DNA replication. That the DNA-negative phenotype of pNTC23 is dominant *in trans* suggests that other functional domains are still present in the mutant proteins.

The most obvious explanation of the dominant negative phenotype of pNTC23 for AAV DNA replication is that the mutant Rep78 and Rep68 proteins have retained their origin-binding ability but have lost their enzymatic activity requiring ATP. Thus, the mutant proteins could compete with the wild-type protein. However, inhibition could also be mediated by protein-protein interactions between mutant and wild-type Rep proteins or by interaction with cellular factors

FIG. 7. Effect of cotransfected mutant AAV genomes on AAV DNA replication. The data were derived from experiments analogous to those in Fig. 4 and 6. The amount of radioactivity hybridized to the RFm species after blotting was determined by cutting the filter and liquid scintillation counting. The effect of cotransfecting genomes is shown by plotting the amounts of radioactivity present in the RFm band as a percentage of the control amount present in adenovirus-infected 293 cells transfected with 0.05 μ g of pNTC244.

important for AAV replication. It is also possible that the dominant phenotype of pNTC23 could be mediated by the mutant Rep52 or Rep40 protein that was also overproduced. This possibility is less likely, however, because a 20-fold excess of pNTC8, which produced mutant Rep52 and Rep40 proteins but not Rep78 and Rep68 proteins, showed no specific inhibitory effect. However, pNTC8 did not overproduce the mutant proteins, so even at a high excess of input genomes it may not generate sufficient mutant protein.

The overproduction of the Rep proteins by pNTC23 provides the first direct evidence that the expression of these proteins is autoregulated. This regulation is complex. We previously reported that Rep78 or Rep68 apparently down-regulates the expression of Rep52 or Rep 40 (29, 38). The present report suggests that Rep78 and Rep68 may also down-regulate their own expression. Preliminary evidence (B. Antoni and B. Carter, unpublished data) suggests that this negative regulation is mediated at least in part at the level of RNA accumulation. Recent evidence also suggests that Rep proteins down regulate the expression of CAT from the p_5 or p_{19} promoter and the expression of Rep mRNA from a simian virus 40 early promoter (3). In contrast, in adenovirus-infected KB cells, Rep proteins apparently in-

FIG. 8. *trans*-Regulation by *rep* of expression from a p_{40} -*cat* plasmid (pTS18) in uninfected 293 cells. Cells were transfected with pTS18 (1 µg) and 10 µg of wild-type (wt) DNA (pNTC244) or 2.5 or 10 µg of mutant DNA (pNTC23 and pNTC3) or 10 µg of control plasmid pBR322 (c). CAT activity was measured 48 h after transfection. The percent acetylation in each reaction is shown at the bottom.

creased the transcription of mRNA from all three AAV promoters (26). Furthermore, we previously showed that for a *cat* gene expressed from an AAV p_{40} promoter in 293 cells, Rep79 or Rep68 simultaneously mediated a modest activation of two- to threefold in p_{40} -*cat* mRNA expression but a fivefold decrease in net CAT activity, apparently because of a more drastic 10- to 20-fold posttranscriptional inhibition of CAT activity. The experiment in Fig. 8 suggests that Rep proteins from pNTC23 may have lost the negative regulation but have retained the positive activation of p_{40} -*cat* expression.

In summary, our results, together with those of others (24, 32a), suggest that the Rep proteins may have separate functional domains, including an *ori* binding domain and probably an ATP binding domain. A preliminary analysis (J. Trempe, R. Owens, N. Chejanovsky, and B. Carter, unpublished data) suggests that the mutant Rep78 protein produced by pNTC23 is a functional AAV *ori* binding protein. Definitive proof of this will require purification and characterization of mutant Rep proteins. Nevertheless, these observations indicate that *rep* may be analogous to other viral genes such as the simian virus 40 T-antigen gene, which has separate domains mediating *ori* binding and ATP binding or ATPase (10–14, 19). Finally, we note that in a variety of protein kinases, not directly related to Rep but containing an ATP binding site, mutation of the conserved lysine specifically alters biological activity. For instance, conversion of lysine 721 of the human epidermal growth factor receptor to alanine abolished tyrosine kinase activity, altered receptor recycling, and abolished signal transduction (23). Similarly, the conversion of lysine 623 of the retroviral mH2 *v-mil* oncogene protein, p100^{gag-mil}, to methionine abolished serine-threonine *in vitro* autophosphorylation and biological properties of the protein (15).

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